Multiple resistance to synthetic auxin herbicides and glyphosate in *Parthenium hysterophorus* occurring in citrus orchards

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ABSTRACT

Dominican farmers have started to apply synthetic auxin herbicides (SAH) as the main alternative to mitigate the impacts of the occurrence of glyphosate resistant (GR) *Parthenium hysterophorus* populations in citrus orchards. A GR *P. hysterophorus* population survived field labeled rates of glyphosate, 2,4-D, dicamba and picloram, which showed poor control (<50%). In *in vivo* assays, resistance levels to glyphosate were high, and moderate to picloram, dicamba and 2,4-D. Sequencing the 5-enolpyruvylshikimate-3-phosphate synthase gene revealed the double Thr-102-Ile and Pro-106-Ser (TIPS) amino acid substitution conferring resistance to glyphosate. Additionally, reduced absorption and impaired translocation contributed to this resistance. Regarding SAH, impaired 2,4-D transport and enhanced metabolism were confirmed in resistant plants. The application of malathion improved the efficacy of SAH (control >50%), showing that metabolism of these herbicides was mediated by cytochrome P450 enzymes. This study reports for the first time, multiple resistance to SAH and glyphosate in *P. hysterophorus.*

Keywords: cytochrome P450 monooxigenase; enhanced metabolism; EPSPS; impaired translocation; ragweed parthenium; reduced absorption; TIPS mutation

1. Introduction

Parthenium hysterophorus L. is one of the most aggressive invasive weeds with high potential of propagation.[1] This species is native to the Gulf of Mexico, Central America, southern North America, the West Indies and Central America, which has become an increasingly problematic weed in many regions.[2] The first cases of resistance in this species to glyphosate were found in Colombia,[3] Dominican Republic and Cuba[4] and recently in Mexico.[5] Due to the uncontrolled occurrence of glyphosate resistant *P. hysterophorus* populations, Dominican farmers have started to apply synthetic auxin herbicides (SAH), either singly or in tank mix with glyphosate, to suppress the glyphosate resistance. However, this weed control strategy, which initially efficiently controlled dicotyledonous weeds, has exerted a high selection pressure selecting for multiple resistance to these herbicides, which has already occurred in *Amaranthus hybridus* and *A. palmeri*.[6,7]

Glyphosate [n-(phosphonomethyl) glycine], applied for the total control of vegetation, is a broad spectrum, non-selective and systemic herbicide.[8] Glyphosate controls weeds by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), disrupting the biosynthesis of aromatic amino acids (phenylalanine, tyrosine and tryptophan).[9] Herbicide resistance can be monogenic or polygenic.[10] Monogenic resistance is governed by genes directly related to the target site through EPSPS mutations and/or EPSPS gene amplification, called target site resistance (TSR) mechanisms.[10-12] Polygenic resistance is regulated by secondary genes not related to the target site gene that cause a reduced absorption and/or translocation of glyphosate, the sequestration of the herbicide in the vacuole, referred to as non-target site resistance mechanisms (NTSR).[13,14]

Synthetic auxin herbicides (SAH) such as 2,4-D (phenoxycarboxylic acid) and dicamba (benzoic acid) are widely used in agriculture to control broadleaf weeds in annual (cereal) and perennial (citrus and orchards) crops, while picloram (pyridine carboxylic acid)) is traditionally used for control of broadleaf weeds in non-agricultural areas.[15,16] SAH act by interfering with auxin-based regulation of plant growth.[17,18] Although SAH have been on the market for 70 years,[19] resistance to these herbicides has been reported in only 38 weed species to date.[20] The occurrence of weeds resistant to SAH is old, but the mechanisms involved in this resistance are poorly understood because its site of action is not well established up to date, and only in the last decade have detailed studies been carried out on this issue.[16,18,21-23] SAH can have several target

sites within plants, potentially different auxin receptor proteins (TIR1 and Auxin F-Box proteins, AFB1–5). The mechanism(s) endowing resistance to SAH have been generally considered to be NTSR,[24] and also because impaired transport was commonly reported.[21,25] Nevertheless, now it is acknowledged that auxin cell influx carrier (AUX1) and auxin efflux carrier proteins (PIN and ABCB proteins) can also be SAH targets in plants.[15] Therefore, very recently TSR mechanisms have started to be reported, associated with a mutation of the AUX1 gene,[22,24] different auxin signaling alterations[26] or with ABCB transporter activity impairing long-distance transport.[21] In the case of SAH, NTSR by means of enzymes that metabolize them is rare.[15] However, enhanced metabolism to 2,4-D was reported in *Papaver rhoeas*,[27] *Amaranthus hybridus*[6] and *A. tuberculatus*,[28] potentially mediated by the cytochrome P450 (Cyt-P450).

In this work, a *P. hysterophorus* population with multiple resistance to glyphosate and SAH, harvested in a citrus orchard from Básima (Dominican Republic) was confirmed directly via *in situ* and greenhouse assays, and the TSR and NTSR mechanisms conferring that multiple resistance were characterized.

2. Materials and Methods

2.1. Chemicals

Trade formulations of 2,4-D, dicamba, picloram and glyphosate were used in field trials and subsequent greenhouse assays (**Table 1**). Both ^{14}C-2,4-D acid and ^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) used for the absorption and translocation assays were analytical grade (> 99.5%).

2.2. Plant material and field trials

Field trials were done during two spring seasons (2015 and 2016) in a cultivated orange crop (*Citrus sinensis*) infested with *P. hysterophorus*, with high resistance to glyphosate (GR), located in Básima, Villa Altagracia, Dominican Republic. In this case, four herbicide treatments (glyphosate, 2,4-D, dicamba and picloram) were used for control effectiveness following a randomized complete block design with four replications, and the experimental unit was a plot of 2 × 15 m (**Table 1**). The herbicides were applied using a Pulverex backpack sprayer equipped with a T with four flat fan nozzles spaced at 50 cm, calibrated to deliver 200 L ha^{-1} at 200 kpa. Forty-five days after treatment (DAT), the visible control to determine the efficacy of the four

herbicides on *P. hysterophorus* was determined using the EWRS (European Weed Research Society) control classification by assigning control scores ranging from 0% (no weed control) to 100% (complete weed destruction).[29] In addition, the reduction of the fresh weight (g m^{-2}) with respect to untreated control were also evaluated.

Mature seeds from at least 5 plants of each experimental unit were collected and stored at 24 ºC. In the spring of 2017, 100 seedlings of the GR population were transplanted into plots (2 x 5 m) in the experimental field at the University of Córdoba, Spain to select for multiple resistance. Plants with 8 true-leaves were treated with 2,4-D at 480 g ae ha^{-1} mixed with picloram at 180 g ae ha^{-1}. Two weeks later, the surviving plants were treated with 720 g ae ha^{-1} of glyphosate mixed with dicamba at 90 g ae ha^{-1}. Finally, surviving plants (~70%) were allowed to grow to maturity and the best mature seed plants with cross- and multiple resistance were harvested and used for the following assays. Seeds of a susceptible (GS) population were also collected in 2017 from a closed area to Básima in which herbicides had never been used.

Seeds of the GR and GS *P. hysterophorus* populations were germinated on moistened filter paper in Petri dishes and placed in a growth chamber at 26/18 °C (day/night) with a photoperiod of 16 h at 850 mmol m^{-2} s^{-1} of light density and 60% relative humidity. Germinated seedlings were transplanted into 8 x 8 x 10 cm pots (one plant per pot) containing a mixture of sand/peat (1:1 v/v) and grown in a greenhouse. Seedlings with 4–6 true-leaves were used for the subsequent experiments.

2.3. Dose response assays. Effect of Malathion.

A first set of GR and GS *P. hysterophorus* plants were treated with different herbicides and doses (2,4-D: 0, 50, 100, 200, 300, 600, 800, 1500, and 2000 g ae ha^{-1}; dicamba: 0, 25, 50, 100, 200, 300, 600 and 1200 g ae ha^{-1}; picloran: 0, 20, 40, 60, 120, 240, 480, 960 and 1920 g ae ha^{-1}; and glyphosate: 0, 15, 30, 60, 120, 240, 480, 960, 1920 and 3840 g ae ha^{-1}). A second set of GR and GS plants were treated with 2000 g ae ha^{-1} of malathion, a potent inhibitor of the cytochrome P450 enzymes (Cyt-P450).[6,27,28] Two h later at 24ºC, plants were only treated with SAH at doses as shown above. Herbicide and malathion applications were conducted in a spray chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzle that delivered 200 L ha^{-1} at the height of 50 cm from plant level. The dose-response assays with or without malathion were repeated twice using six plants per herbicide rate. Aboveground fresh weight per plant was determined 21 DAT, and data

were expressed as the percentage of the untreated control. Herbicide rates inhibiting the plant growth by 50% with respect to the untreated control (GR_{50}) were determined for each population, and the R/S ratio (FR) was computed as GR_{50} (GR)/GR_{50} (GS).[6]

2.4. ^{14}C-2,4-D and ^{14}C-glyphosate absorption and translocation

The ^{14}C-2,4-D and ^{14}C-glyphosate absorption and translocation were evaluated as described by Rey-Caballero et al.[23] and Bracamonte et al.[6] Both ^{14}C-herbicides were mixed with their respective trade formulation until achieving a final concentration of 480 g ae ha^{-1} and 800 g ae ha^{-1} of 2,4-D and glyphosate, respectively. In both cases, the specific activity was 0.834 kBq μL^{-1}. Two set of plants with 4 true-leaves of GR and GS populations (herbicides were evaluated separately) were treated with a drop (1 µL $plant^{-1}$) of radiolabeled solution on the adaxial surface of the second youngest leaf, in a completely randomized design. There were five replications and each experiment was arranged in a completely randomized design and the experiments repeated twice.

At 96 h after treatment (HAT), the unabsorbed ^{14}C-herbicides were removed with 50% (v/v) methanol (2,4-D) or acetone (glyphosate) by washing the treated leaf three times (1 mL of washing solution each). The rinse solution was mixed with 2 mL of scintillation liquid (Ultima Gold, Perkin-Elmer, BV BioScience Packard) and measured by liquid scintillation spectrophotometry (LSS) using a scintillation counter (Beckman LS 6500, Beckman Coulter Inc., Fullerton CA). The whole plants were removed from the pot and sectioned into treated leaves, remainder of the plant and roots. The plant sections were individually stored in cellulose cones for combustion, dried at 60 °C for 96 h and burned using a biological oxidant (Packard Tri Carb 307, Perkin-Elmer, Waltham, MA). The CO_2 released from combustion was captured in 18 mL of a mixture of Carbo-Sorb E and Permafluor (1:1 v/v) (Perkin-Elmer, BV BioScience Packard). The radioactivity of each sample was measured for 10 min by LSS. The radioactive values of absorption and translocation of ^{14}C were expressed as a percentage of the total ^{14}C-herbicide applied and recovered, respectively.

2.5. 2,4-D and glyphosate plant metabolism

Ten plants with four true leaves of GS and GR *P. hysterophorus* populations were used for the 2,4-D metabolism study, following the methodology described by Torra et al.[27] using a Gold LC System from Beckman Coulter (Fullerton, USA) equipped with a diode array detector (DAD) detector (wavelength range 190–600 nm). Plants were treated with 600 g ae ha^{-1} of 2,4-D and harvested at 96 HAT. The

chromatographic separation was carried out using a Kinetex® EVO C18 column (150 mm, 4.6 mm id, 2.6μm particle size) from Phenomenex Inc. (Torrance, CA, USA), furnished with a 4.6 mm SecurityGuard^{TM} ULTRA cartridges. Quantification of 2,4-D and its metabolites was based on the calibration curve of 2,4-D.

For glyphosate, other set of ten plants (4 true leaves) per populations were treated with glyphosate at 360 g ae ha^{-1} and cut 96 HAT. The extraction and determination of glyphosate and its metabolites were done following the methodology described by Rojano-Delgado et al.[30] using an Agilent G1600A 3D capillary electrophoresis instrument equipped with a DAD (wavelength 190-600 nm).

In each case, a set of untreated plants was reserved as control and results were expressed as percentage of 2,4-D or glyphosate and their respective metabolites.

2.6. EPSPS activity

For the EPSPS activity study, five grams of young leaf tissue of GS and GR populations were used following the protocol described by Dayan et al.[31] An EnzChek Phosphate Analysis Kit (Invitrogen, Carlsbad, CA, USA) was used to determine the EPSPS activity in presence and absence (basal activity) of glyphosate. The total content of protein in crude extract was measured at 595 nm with the Bradford colorimetric method.[32] The glyphosate concentrations used to determine the inhibition of enzymatic activity (I_{50}) were 0, 0.1, 1, 10, 100, 500 and 1000 μM. The amount of inorganic phosphate (μmol) released was measured for 10 min at 360 nm in a spectrophotometer (DU-640, Beckman Coulter Inc. Fullerton, USA). The EPSPS activity was calculated by determining the amount of phosphate (μmol) released μg of total soluble protein (TSP)$^{-1}$ min^{-1}. Three replicates per population and glyphosate concentration were used.

2.7. Partial sequencing of the EPSPS gene

One hundred mg of young leaf tissue of R and S populations were used to obtain total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. CA, USA. The primers (forward: 5′- GGTTGTGGYGGTVTRTTTCC-3′ and reverse: 5′-GTCCCAASTATCACTRTGTTC-3′) and PCR conditions (total volume of 25 μL per reaction) used in this study were those described by Alcántara-de la Cruz et al.[11] PCR products (2-3 plant^{-1} from 10 plants per populations) were sequenced (STAB VIDA, Caparica, Portugal). cDNA sequences were assembled searching for the commonly

reported mutation at 102 and 106 positions[10,11] using the Muscle algorithm incorporated into SeqMan Pro 11 (DNASTAR; Wisconsin, USA).

2.8. Statistical analysis

The amount of herbicide causing the fresh weight reduction (GR_{50}) (glyphosate, 2,4-D, dicamba and picloram) and EPSPS inhibition (I_{50}) (only for glyphosate) by 50% were determined from the data of dose-response and EPSPS enzyme activity assays. Data were subjected to non-linear regression analysis using the log-logistic equations[33]: $Y = c + \{(d-c)/[1 + (x/g)^b]\}$ (three-parameters for GR_{50}) and $Y = c + \{(d-c)/[1 + (x/g)^b]\}$ (four-parameters for I_{50}). In these equation, *Y* is the percentage of fresh weight reduction or EPSPS inhibition relative to the control; c and d are the lower and upper limits, respectively, of the curve; b is the slope at the inflection point (i.e., GR_{50} or I_{50}); and x is the herbicide dose. The three-parameters model assumes that the lower limit is zero. Analyses were conducted using the *drc* package with program R version 3.2.554, and the data were plotted using SigmaPlot 11.0 (Systat Software, Inc., USA).

Data of ^{14}C-herbicides absorption and translocation, 2,4-D and glyphosate metabolism were subjected to ANOVA using Statistix version 9.0 from Analytical Software (Tallahassee, FL, USA). Model assumptions of normal distribution of errors and homogeneous variance were graphically inspected. When necessary, the means were compared using Tukey's test's at the 95% probability level.

3. Results and discussion

3.1. Field trials

The GR *P. hysterophorus* population showed low injury and high survival rates in the field to the different treatments with SAH and glyphosate. Farmers consider that a minimum of 80 to 85% mortality is necessary to have satisfactory control.[30,34] However, after 45 DAT, the control level of the GR *P. hysterophorus* were not greater than 20% in the cases of glyphosate and 2,4-D compared to untreated plots. Dicamba and picloram showed a slightly better control (31 and 43%, respectively) (**Table 2**). These results revealed multiple and cross-resistance to glyphosate and SAH for the first time in this species. Few cases of this type of multiple resistance have been confirmed in the world.[6,20] The experimental recurrent selection allowed obtaining individuals with multiple resistance to the four herbicides. The effect of the first herbicide mixture (2,4-D + picloram) was very rapid, during the first 2-5 DAT, as the plants stopped growing

and classical uncontrolled tissue growth and epinasty appeared, followed by growth inhibition and death of some individuals. However, most of the GR plants recovered their normal growth 14 DAT. Treatment with glyphosate and dicamba only caused chlorosis in some plants at 10 DAT, but ~70% plants survived both treatments.

3.2. Dose response assays. Effect of Malathion.

In the dose-response assay (greenhouse), the GS *P. hysterophorus* population was well controlled with no surviving plants at field doses of glyphosate, 2,4-D, dicamba and picloram. However, more than 90% of GR population survived at field rates or higher of the above herbicides. Based on the GR_{50} values, the resistance factor (RF) estimated for glyphosate was higher than those found for the SAH (**Table 3**). The GR plants, treated with glyphosate, presented a high value of GR_{50} of 4251.59 g ae ha^{-1}, while the SAH 2,4-D, dicamba and picloram had values lower than 520.71, 425.14 and 381.35, respectively (**Figure 1, Table 3**). Therefore, the multiple and cross-resistance to glyphosate, 2,4-D, dicamba and picloram was evident in this population from Dominican Republic.

In a second experiment applying malathion 2-h before the application of herbicides, the RF ratios of 2,4-D and dicamba were reduced from 5.1 to 3.2 and from 5.3 to 2.4, respectively, evidencing a clear inhibition, but not total, of the Cyt-P450 enzymatic complex. However, malathion did not reduce the resistance level to picloram (**Figure 1, Table 3**). These results show that two possible mechanisms, reduced translocation and enhanced metabolism, could be involved in the resistance of *P. hysterophorus* to 2,4-D and dicamba, as observed in *Papaver rhoeas*[27] and *Raphanus raphanistrum*.[21]

3.3. ^{14}C-2,4-D and ^{14}C-glyphosate absorption and translocation

There were no differences between GR and GS *P. hysterophorus* plants in the amount of ^{14}C-2,4-D absorbed via foliar (70-76%) at 96 HAT. On the contrary, the GS population absorbed 18% more ^{14}C-glyphosate than the GR population (90 versus 72%). Regarding translocation, both for ^{14}C-2,4-D and ^{14}C-glyphosate, the GS population moved more ^{14}C-herbicide from the treated leaf to the remainder of the plant and roots than the GR population at 96 HAT (**Table 4**).

Taking into account the data obtained in the dose response trials with plants treated with malathion, we can deduce that the resistance to 2,4-D shown by the GR population could be due to two simultaneous NTSR mechanisms, first a lower

translocation of the herbicide and second an enhanced herbicide metabolism, where Cyt-P450 could be involved. In the case of glyphosate, we can only infer that the lower absorption and less translocation of herbicide contributed to the resistance observed in the GR population. The impaired transport from the treated leaf to meristematic growing points reduces the efficacy of systemic herbicides.[35,36] However, the high resistance levels to glyphosate can not only be explained with the association of these mechanisms. Therefore, other mechanisms such as metabolism (NTSR) and/or mutations in the EPSPS and number of copies and overexpression (TSR) could also be involved, as observed in other *P. hysterophorus* populations collected in Cuba, Dominican Republic[4] and Mexico.[5] In the case of 2,4-D, our results are in agreement with those observed for *Raphanus raphanistrum*[15,21] and *Papaver rhoeas*.[23,27]

3.4. 2,4-D and glyphosate plant metabolism

The 2,4-D metabolism confirmed that this resistance mechanism was present in the GR population, but not in the GS population. Indeed, ~87% of the herbicide (toxic form) was transformed into metabolites (non-toxic forms) ascribed as 2,3-D (~47%), 2,5-D (~23%) and conjugated-metabolites (~8%) in the GR population according to retention times (**Table 5**). These metabolism patterns were similar to those observed in SAH resistant *Papaver rhoeas* populations.[27] The Cyt-P450 may be responsible for this enhanced metabolism of 2,4-D, because since the activity of this enzyme complex was reduced in the doses response assays when malathion was applied.

On the other hand, glyphosate metabolism did not seem to be a resistance mechanism against this herbicide. In both GR and GS populations, more than 93% of glyphosate remained as toxic form within the plant, and ~7% was transformed into AMPA and glyoxylate (**Table 5**). Some authors consider that metabolism has a low contribution to the resistance or, even more, that it is nonexistent.[37,38]

3.5. Basal EPSPS activity

The basal EPSPS activity ranged from 0.13 to 0.16 μmol Pi μg^{-1} TSP min^{-1}, with no differences between GS and GR *P. hysterophorus* populations (**Figure 2A**). Conversely, the EPSPS inhibition occurred at different glyphosate concentrations (**Figure 2B**). The I_{50} values of the GS and GR populations were 9.8 and 514 μM glyphosate, respectively, i.e., the GR population required at least 50 times more glyphosate to inhibit EPSPS by 50% than the GS population.

The *EPSPS* gene duplication causes a greater expression of this gene and consequently a massive overproduction into active enzyme.[39] The enzyme is sensitive to glyphosate, but since there is a higher concentration in R plants, that carry this resistance mechanism, a fraction of the enzyme faces the glyphosate acting as a sponge, and the another fraction develops its normal metabolic functions in the shikimic pathway.[40,41] *EPSPS* gene duplication has been reported as the major resistance mechanism conferring resistance in several glyphosate resistant weed species.[42-44] However, the results of the EPSPS basal activities allowed us discard the contribution of this resistance mechanism in the GR *P. hysterophorus* populations. On the other hand, the differential EPSPS inhibition rate of the GR plants, exposed at increasing glyphosate concentrations, suggested that the interaction of the herbicide with its target-site was due to changes in the tridimensional conformation of the enzyme, as pointed out in previous studies.[10,12,45]

3.6. EPSPS gene sequencing

The sequencing of the conserved region of the *EPSPS* gene, including the positions ^{95}LFLGNAGTAMRPL^{107},[41,45] showed a double amino acid substitution at positions 102 and 106 from Thr to Ile and from Pro to Ser, respectively, in the GR *P. hysterophorus* population. This combination, Thr-102-Ile and Pro-106-Ser, is known as TIPS.[46] The codons ACT^{102} and CCG^{106} encoded for Thr and Pro, respectively, in the GS population, as expected (**Figure 3**).

The appearance of the TIPS mutation in the GR *P. hysterophorus* population explained the high levels of glyphosate resistance observed in these plants. Previously, only the Pro-106-Ser mutation was reported in this species.[4,5] This mutation provides low resistance levels and the Thr-102-Ile high resistance levels.[45,46] However, while the first substitution preserves EPSPS functionality, the second one reduces the affinity of EPSPS to its substrate (PEP, phosphoenolpyruvate).[38] Due to this loss of affinity in the binding between the glyphosate/PEP versus EPSPS enzyme, mutation at 102 position would be unlikely to occur first or independently of Pro-106 mutations and appear generally associated with changes in the position 106.[36] However, the single mutation Thr-102-Ser was found in *Tridax procumbens*.[12] *Partenium hysterophorus* represents the third species worldwide (second broadleaf) carrying the TIPS mutation, after its previous identification in *Bidens pilosa* and *Eleusine indica*.[11,46,47] In addition, the novel

double TIPT (Thr102Ile and Pro106Thr) amino acid substitution conferring high resistance to glyphosate was found in *B. subalternans*.[48]

Cross resistance to three different chemical families of SAH is described for the first time in *P. hysterophorus* worldwide. Both impaired transport and enhanced metabolism by means of Cyt-P450 could be the NTSR mechanisms responsible of the observed resistance to SAH in this species. Additionally, this population was also multiple resistant to glyphosate. Reduced absorption and impaired translocation contributed partially to the glyphosate resistance of this GR *P. hysterophorus* population as NTSR mechanisms. In addition, the double amino acid substitution TIPS, from Pro to Ser occurring at the 106 position, and from Thr to Ile in the 102 position of the EPSPS gene, increased the resistance to this herbicide in the population as TSR mechanisms. This study describes for the first time, not only the cross- and multiple resistances to SAH and glyphosate, but also the double gene mutation TIPS, in a *P. hysterophorus* population from the citrus producing region of the Dominican Republic.

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522 **Table 1**. Herbicides tested in field and greenhouse experiments for control effectiveness
523 of *Parthenium hysterophorus* multiple resistant.

Herbicide	Trade name	MOA/HRAC	Chemical group	Application time/Action
2,4-D	Esteron 60%	Synthetic auxin/O	Phenoxycarboxylic acid	Post-emergence/ systemic
Dicamba	Bambel D 48%	Synthetic auxin/O	Benzoic acid	Post-emergence/ systemic
Picloram	Picloram Nova 27.7%	Synthetic auxin/O	Pyridine carboxylic acid	Post-emergence/ systemic
Glyphosate	Roundup Ultimate 48%	EPSPS/G	Glycine	Post-emergence/ strong systemic

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Table 2. Control level (%) and fresh weight reduction with glyphosate and synthetic auxins herbicides on a glyphosate resistant *Parthenium hysterophorus* population over two years (2015 and 2016) infesting a citrus field from Básima, Dominican Republic

Herbicide	Field doses[a]	Control (%)[b]	Fresh weight (g/m^2)
2,4-D	480	17.5±3.8	395±4.7
Dicamba	90	31.5±2.9	305±3.7
Picloram	180	43.6±1.5	260±7.3
Glyphosate	800	17.5±3.4	431±7.1
Control	0	0	416±11.9

[a] given in g ae ha^{-1}; [b] 0= no weed control, and 100= complete weed destruction. Evaluations were performed 45 days after herbicide treatments.

533 **Table 3.** Estimated values of GR_{50} (herbicide rate reducing the fresh weight by 50%)
534 and resistance factor (RF) of synthetic auxin herbicides in the absence (-) or presence
535 (+) of malathion and glyphosate alone in *Parthenium hysterophorus* populations
536 resistant (GR) and susceptible (GS) to glyphosate

Herbicide	Population		d	b	R^2	GR_{50}	RF
2,4-D	(-Malation)	GR	99.9	4.2	0.998	520.7 ± 1.9	5.1
		GS	99.1	3.8	0.999	102.2 ± 1.9	1.0
	(+Malation)	GR	98.3	2.5	0.989	322.8 ± 4.6	3.2
		GS	98.6	4.3	0.99	99.7 ± 3.8	1.0
Dicamba	(-Malation)	GR	100.7	0.3	0.998	425.1 ± 1.8	5.3
		GS	99.6	2.2	0.999	79.6 ± 1.6	1.0
	(+Malation)	GR	99.5	0.7	0.999	188.7 ± 1.4	2.4
		GS	99.9	1.6	0.999	66.2 ± 0.7	0.8
Picloram	(-Malation)	GR	99.9	0.1	0.999	381.4 ± 1.2	6.8
		GS	100.4	0.4	0.996	56.0 ± 2.5	1.0
	(+Malation)	GR	96.7	1.2	0.99	375.7 ± 3.5	6.7
		GS	101.1	2.6	0.99	54.2 ± 3.8	1.0
Glyphosate	GR		99.8	1.6	0.999	4251.6 ± 0.6	49.8
	GS		95.1	4.0	0.99	85.5 ± 4.3	1.0

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538

Table 4. Absorption and translocation of ^{14}C-2,4-D and ^{14}C-glyphosate in *Parthenium hysterophorus* populations resistant (GR) and susceptible (GS) to glyphosate at 96 hours after treatment

Herbicide	Population	Absorption[a]	Translocation[b]		
			Treated leaf	Rest of Plant	Root
2,4-D	GS	76.6±11.8 a	72.8±10.1 b	19.4±13.3 a	8.2±7.1 a
	GR	70.1±15.4 a	88.2±12.3 a	9.8±0.4 b	2.6±1.5 b
glyphosate	GS	90.2±3.1 a	34.2±5.4 b	31.0±5.4 a	30.8±1.7 a
	GR	72.2±4.1 b	71.6±6.1 a	16.2±2.6 b	12.8±1.3 b

[a]Percent from the applied ^{14}C-herbicide. [b]Percent from the absorbed ^{14}C-herbicide. Same letter within a column are not different by the Tukey test at 95%. ± Standard error of the mean (n= 5) for each herbicide treatment.

547 **Table 5.** Herbicides and their respective metabolites, relative percentage in *Parthenium*
548 *hysterophorus* populations resistant (GR) and susceptible (GS) to glyphosate at 96 hours
549 after treatment

Population	2,4-D	2,3-D	2,5-D	Conjugated-
GR	13.3 ± 7.3 B	46.5 ± 8.1	22.5 ± 7.8	8.1 ± 3.9
GS	99.5 ± 6.55 A	nd	nd	nd
	Glyphosate	AMPA	Glyoxylate	Sarcosine
GR	93.5 ± 8.1 A	2.8 ± 0.1 A	4.8 ± 2.4 A	nd
GS	92.6 ± 3.3 A	2.3 ± 0.25 A	5.5 ± 1.7 A	nd

550 nd: non-detectable. AMPA: aminomethylphosphonic acid. Same letter within a column
551 and herbicide are not different by the Tukey test at 95%. ± Standard error of the mean
552 (*n*= 10) for each herbicide treatment.
553
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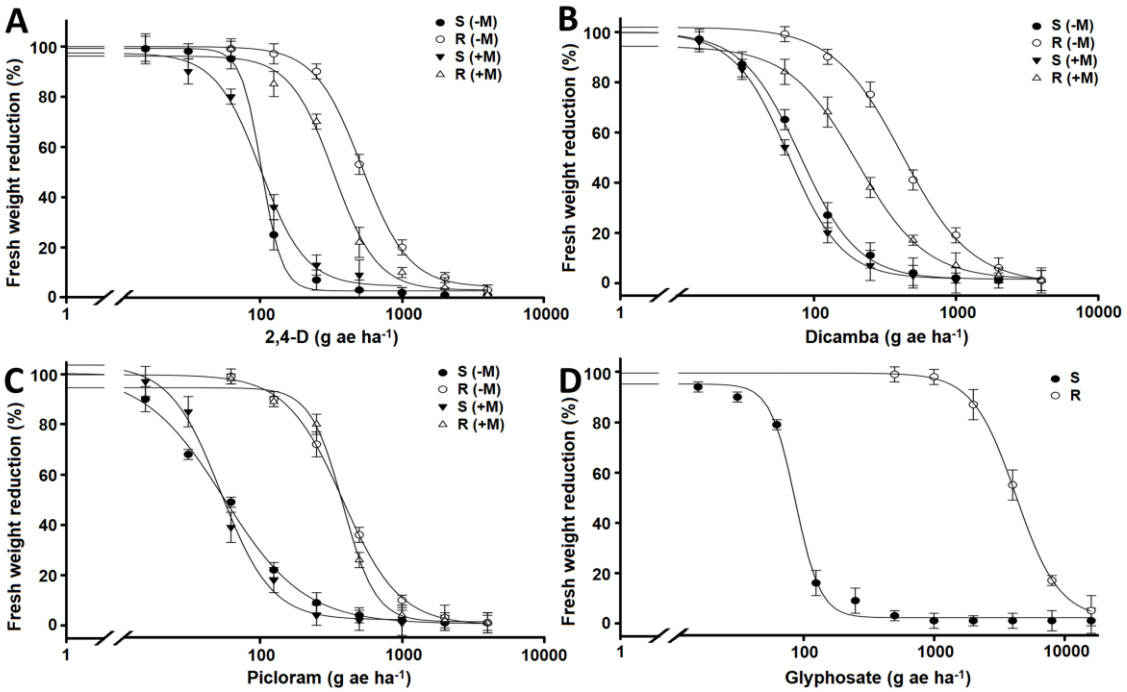

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556 **Figure 1.** Dose–response curves of susceptible (S) and resistant (R) *Parthenium*
557 *hysterophorus* populations to 2,4-D (A), dicamba (B) and picloram (C) in the absence (-
558 M) and presence (+M) malathion and glyphosate alone (D). Data were expressed as
559 percentage of the mean fresh weight of untreated control plants.

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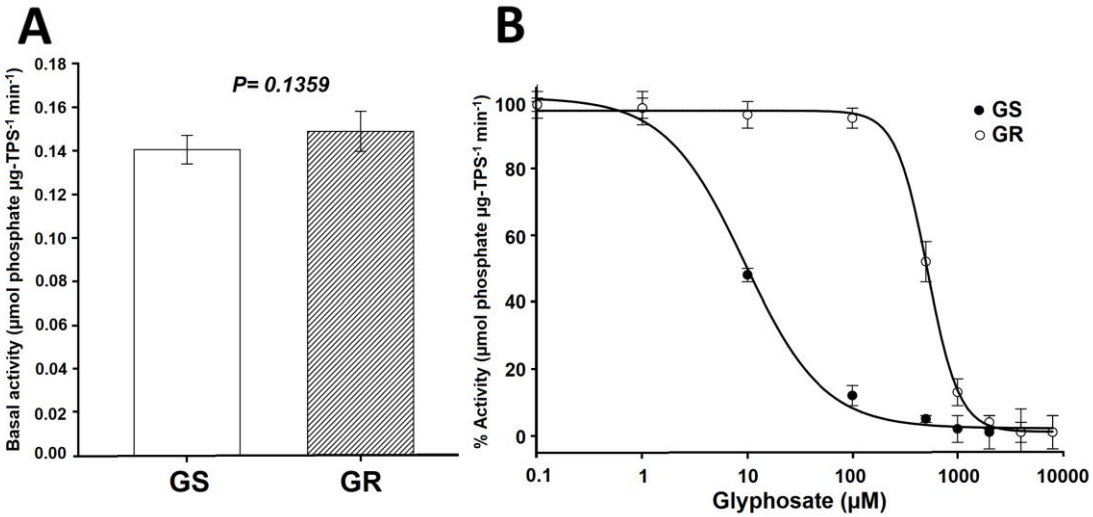

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563 **Figure 2.** Activity of the total soluble protein (TPS) 5-enolpyruvylshikimate-3-
564 phosphate synthase (EPSPS) in *Parthenium hysterophorus* GS and GR populations,
565 collected in citrus. A) Basal EPSPS activity (absence of glyphosate). Histograms
566 represent the means and vertical bars the standard error (*n*= 5). B) Dose-response curves
567 of the EPSPS enzyme activity, expressed as percentage of the untreated control,
568 exposed at different glyphosate concentrations (µM). Vertical bars represent the
569 standard error of the mean (*n*= 5).

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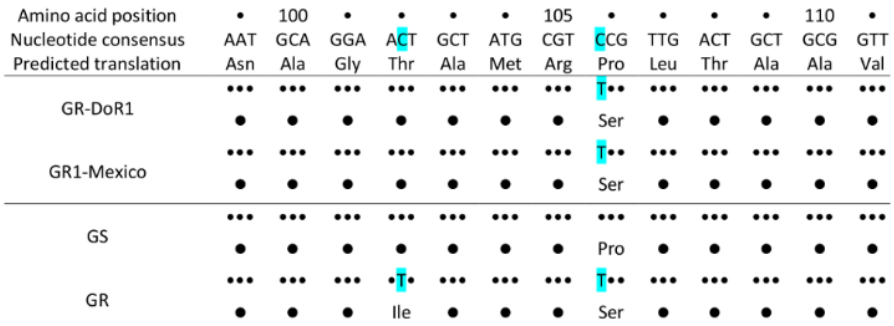
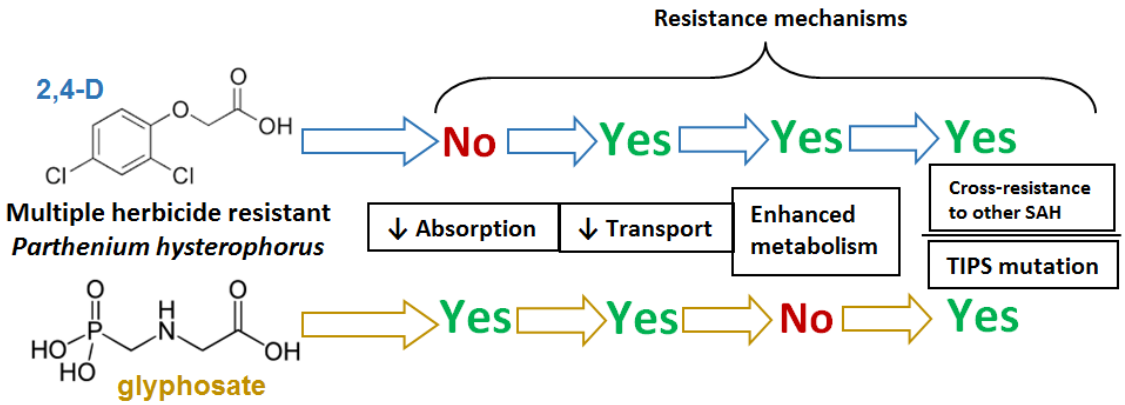


Figure 3. Partial alignment of nucleotides of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene of the GR and GS *Parthenium hysterophorus* populations, compared with two glyphosate-resistant population from Dominican Republic (Do)[4] and Mexico.[5]

580 **Table of contents graphic**

581



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